

# The absence of p53 is critical for the induction of apoptosis by 5-aza-2'-deoxycytidine

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**The absence of functional p53 has complex consequences on the cellular responses to cytotoxic drugs. Here, we have examined the role of p53 in the response to 5-aza-2'-deoxycytidine (5-aza-dC or decitabine). Primary mouse embryonic fibroblasts deficient for p53 undergo apoptosis after treatment with 5-aza-dC. When compared with other demethylating drugs or chemotherapeutic treatments, 5-aza-dC showed the highest selectivity ratio for triggering apoptosis in p53-deficient cells relative to wild-type cells. Moreover, the apoptotic efficacy of 5-aza-dC is proprietary of p53-deficient cells, not being observed in cells lacking other cell-cycle regulators, such as p19<sup>ARF</sup>, p16<sup>INK4a</sup>, p21<sup>CIP1/WAF1</sup>, E2F-1, or E2F-2. Interestingly, treatment with 5-aza-dC results in the same degree of global genomic hypomethylation in wild-type and p53-null cells. However, wild-type cells activate p53 and arrest at G2/M, whereas p53-null cells accumulate severe chromosomal aberrations and undergo apoptosis. Significantly, the impact of p53-deficiency on the response to 5-aza-dC is not exclusive of primary non-neoplastic cells, but it is also present in neoplastically transformed cells. Finally, treatment of mice bearing genetically defined tumors with nontoxic doses of 5-aza-dC results in therapeutical responses only on tumors lacking p53, but not on tumors lacking p19<sup>ARF</sup>. Together, our results put forward the hypothesis that the absence of p53 may determine a higher chemotherapeutic index for 5-aza-dC.**

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## Introduction

There is an increasing interest in the use of DNA methylation inhibitors as chemotherapeutic agents for cancer; however, a detailed understanding of the

consequences of DNA demethylation is still lacking. The nucleoside analogue 5-aza-2'-deoxycytidine (5-aza-dC) has been widely used as a DNA methylation inhibitor (Jones and Taylor, 1980), and it is being currently tested in clinical trials for a number of malignancies, manifesting a particularly high efficiency against acute myeloid leukemia, advanced myelodysplastic syndromes, and chronic myeloid leukemias in blastic phase (Santini *et al.*, 2001). 5-Aza-dC is incorporated into the DNA of replicating cells in substitution of deoxycytidine rendering the DNA refractory to methylation, but susceptible to form covalent adducts with the DNA methyltransferases (Juttermann *et al.*, 1994; Christman, 2002). As a consequence, 5-aza-dC results in the reactivation of genes silenced by aberrant promoter methylation, in global demethylation of the genome, and in severe DNA lesions. Other nucleoside analogues aimed to interfere with DNA methylation are also under investigation, including some that are not incorporated into the DNA and therefore are less genotoxic (De Cabo *et al.*, 1994; Lin *et al.*, 2001). However, despite the wealth of information on DNA methylation inhibitors, systematic studies aimed at elucidating the genetic factors that influence the chemotherapeutic response to these inhibitors are lacking.

Approximately, half of the human cancers have deleted or mutated p53 (Hollstein *et al.*, 1991). This high percentage of incidence makes very attractive the design of chemotherapeutic regimes that could exploit the biological consequences of lacking p53. However, the impact of p53 in the sensitivity to chemotherapy is complex (Peller, 1998; Weller, 1998; Gudkov and Komarova, 2003). This complexity is a reflection of the functioning of p53 as a DNA damage checkpoint. In particular, while the absence of p53 increases the overall resistance to apoptosis, at the same time, it allows proliferation in the face of genomic instability, thus leading to mitotic catastrophe and cell death. This last scenario is supported by a number of reports describing that p53-deficient cells are hypersensitive to apoptosis induced by a variety of chemotherapeutic drugs, such as paclitaxel (Hawkins *et al.*, 1996), UV light (Lackinger and Kaina, 2000; Smith *et al.*, 2000; McKay *et al.*, 2001),  $\gamma$ -irradiation (Lips and Kaina, 2001), cisplatin (Hawkins *et al.*, 1996; McKay *et al.*, 2001), and alkylating agents

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(Lackinger and Kaina, 2000). Conceivably, it is the balance between resistance to apoptosis and permissivity to genomic instability what finally determines the impact of p53 on chemotherapeutic responses. This balance will certainly depend on the cellular context as well as on each particular chemotherapeutic agent.

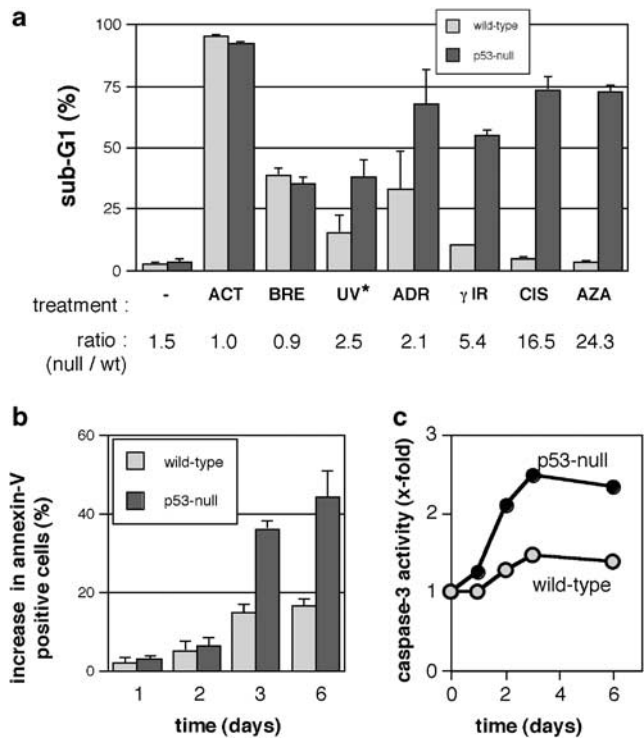
Here, we have used murine fibroblasts with genetically defined mutations to explore the role of p53 in response to methylation inhibitors. In particular, we have studied cells individually lacking either p53 or related cell-cycle regulators and tumor suppressors, such as p19<sup>Arf</sup>, p21<sup>Cip1/Waf1</sup>, E2F-1, and E2F-2, and also in cells rendered tumorigenic by the introduction of oncogenic Ras. Collectively, our results identify p53 as a critical determinant of the cellular response to 5-aza-dC.

## Results

### *p53-Deficient mouse embryo fibroblasts (MEFs) are hypersensitive to 5-aza-dC*

As a first approximation to analyse the cytotoxic effect of demethylating drugs, we began by framing this effect into the context of other chemotherapeutic drugs and radiation treatments. For this, we compared in parallel the apoptotic effect of wild-type and p53-deficient primary MEFs. We analysed the level of apoptosis 6 days after treatments were initiated because long-term effects are conceivably more relevant for chemotherapy than short-term ones. A total of seven chemotherapeutic treatments were tested, and the ratio between the percentage of apoptosis in p53-null and wild-type MEFs was calculated (Figure 1a). Some treatments (actinomycin D, brefeldin A, UV, and adriamycin) had similar apoptotic efficacy on MEFs with or without p53 (selectivity ratios ranging from 0.9 to 2.5). Other treatments presented an enhanced effect on p53-null cells, as was the case of  $\gamma$ IR (ratio 5.4), cisplatin (ratio 16.5), and, even more selectively, 5-aza-dC (ratio 24.3). The hypersensitivity of p53-null cells to cisplatin serves as a positive control, in agreement with previous reports that have studied the impact of p53 on the sensitivity of human cells to cisplatin (Hawkins *et al.*, 1996).

Time course experiments to determine the kinetics of the cell death induced by 5-aza-dC indicated that death was maximal between 3 and 6 days after initiating the treatment (data not shown), which is in agreement with the time required by this drug to exert its full demethylating effect (Haaf, 1995). The cytotoxicity of 5-aza-dC was further analysed to determine if it indeed showed characteristic features of apoptosis. In particular, we observed that p53-null cells treated with 5-aza-dC lost the asymmetry of the cytoplasmic membrane, as manifested by the binding of annexin-V (Figure 1b), and contained activated caspase-3-like (Figure 1c). Together, these data indicate that the absence of p53 has a profound impact on the apoptotic efficacy of the demethylating drug 5-aza-dC, and this novel finding



**Figure 1** p53-deficient MEFs are hypersensitive to 5-aza-dC. (a) Cell death was measured as the percentage of cells with sub-G1 DNA content, 6 days after exposure of cells to the indicated treatments. At the bottom is indicated the ratio between the percentages of cell death observed in primary wild-type and p53-null MEFs, respectively. The different treatments were as follows: 5  $\mu$ g/ml brefeldin-A (BRE); 1  $\mu$ g/ml actinomycin-D (ACT); 9 J/m<sup>2</sup> UV-C (UV), the asterisk is to indicate that in this particular case the analysis was done 3 days after treatment because at 6 days cells had resumed growth and apoptosis was not evident; 0.5  $\mu$ g/ml adriamycin (ADR); 20 Gy  $\gamma$ -radiation ( $\gamma$ IR); 5  $\mu$ g/ml cisplatin (CIS); and 10  $\mu$ M 5-aza-dC (AZA). The values correspond to the average and s.d. of three parallel assays, each one with cell cultures from independent embryos. (b) Induction of apoptosis by 5-aza-dC measured as the percentage of annexin-V-positive cells. Apoptosis was measured at the indicated times in primary MEFs of the indicated genotype. The values correspond to the increase in the percentage of annexin-V-positive cells with respect to nontreated cells. The values correspond to the average and s.d. of three parallel assays, each one with cell cultures from independent embryos. (c) Activation of caspase-3 upon treatment with 10  $\mu$ M 5-aza-dC. Caspase-3 activity was measured with a fluorescent substrate. Wild-type and p53-null MEFs had similar basal levels of caspase-3 activity (arbitrary units of fluorescence: 125 for wild type and 106 for p53-null). The values indicate the ratio of caspase-3 activity relative to nontreated cells. Each value is the average of a duplicate measurement

constitutes the basis to further investigate the underlying mechanisms.

### *Demethylation induced by 5-aza-dC is not the primary cause of cell death*

Demethylating drugs can be grouped in two classes according to their mechanism. Nucleoside analogues, such as 5-aza-dC and zebularine, are incorporated into the DNA of replicating cells, where they result in the formation of covalent complexes with DNA

methyl-transferases (DNMTs) (Christman, 2002; Cheng *et al.*, 2003). On the other hand, non-nucleoside compounds, such as procainamide or *S*-adenosyl-homocysteine (an analogue of the universal methyl-donor metabolite *S*-adenosylmethionine), inhibit DNMT enzymatic activity without being incorporated into DNA (Ueland, 1982; Scheinbart *et al.*, 1991). We have compared the efficacy of the aforementioned drugs in triggering cell death. Only 5-aza-dC and, to a lower extent, zebularine were effective in inducing apoptosis (Figure 2a). However, the selectivity of zebularine for p53-deficient cells was not as marked as in the case of 5-aza-dC.

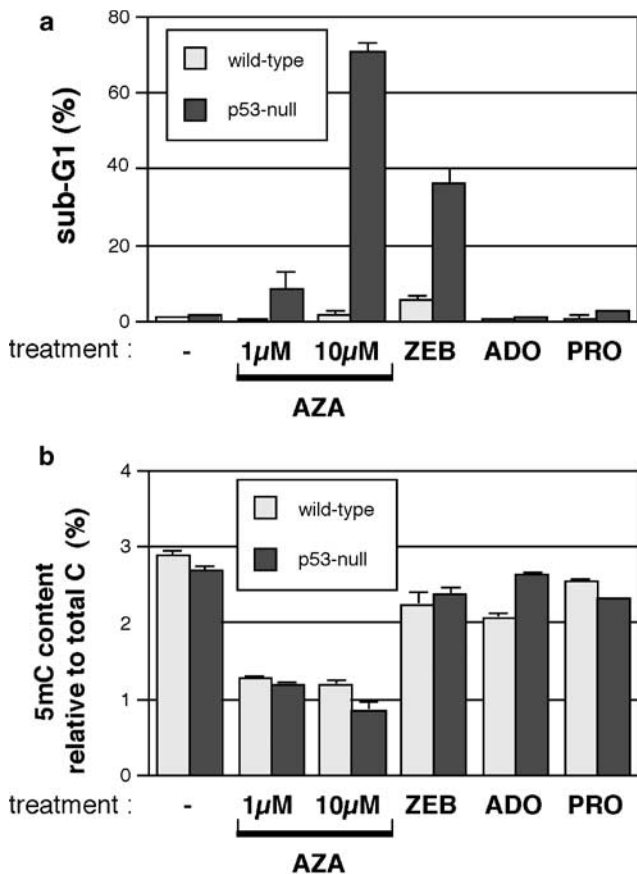
The effect of the four demethylating drugs in producing global genomic demethylation was compared, both in wild-type and p53-deficient MEFs. Total 5-methyl-cytosine (5mC) content was measured by high-

performance capillary electrophoresis 3 days after initiating the treatments with the demethylating drugs. We observed that 5-aza-dC was significantly more efficient in demethylating the genome than the other three drugs (Figure 2b). Notably, the demethylating effect of 5-aza-dC was quantitatively similar in wild-type and p53-deficient cells (about 60% decrease in 5mC content), indicating that the degree of demethylation is not influenced by the status of p53. Moreover, it is informative to note that the extent of demethylation is similar at 1 and 10  $\mu$ M 5-aza-dC concentrations (Figure 2a), which is in contrast with the fact that 1  $\mu$ M 5-aza-dC is inefficient in triggering apoptosis in p53-null cells (see Figures 2a and 3a). Together, these observations suggest that the effects of 5-aza-dC are not dictated simply by the degree of demethylation. In agreement with this, other investigators have concluded previously that the cytotoxic effects of 5-aza-dC are related primarily to the chromosomal damage produced by the formation of adducts between DNMTs and DNA (Juttermann *et al.*, 1994).

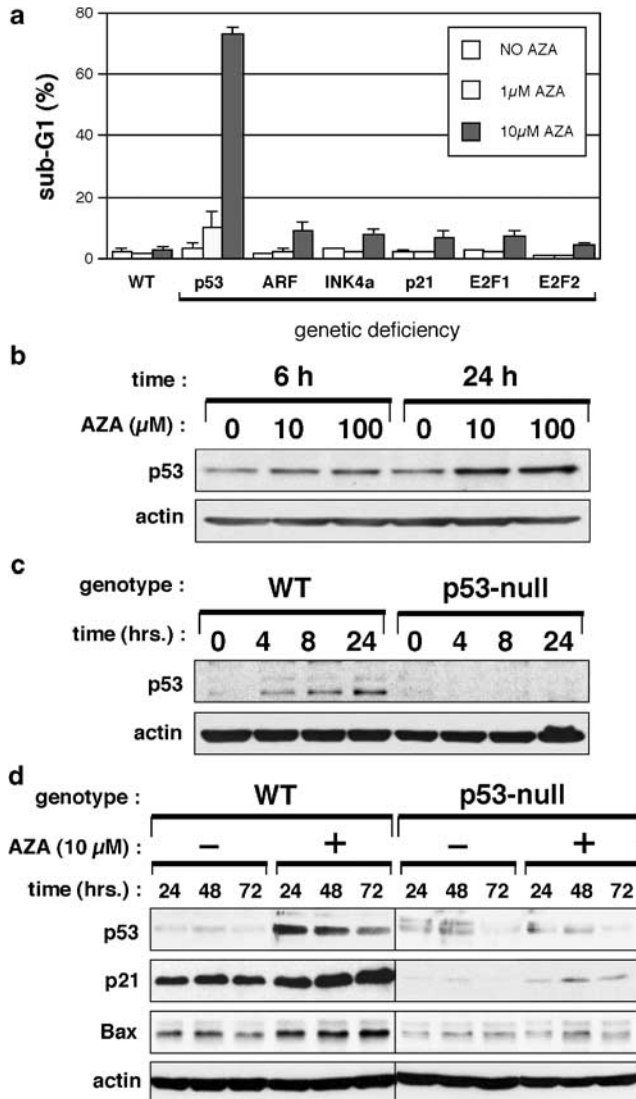
#### 5-Aza-dC induces a G2/M arrest specifically dependent on p53

To evaluate the specificity of the apoptotic response observed in p53-deficient cells, we treated a series of primary MEFs lacking different cell-cycle regulators with 5-aza-dC. In particular, we tested MEFs deficient for p19<sup>ARF</sup>, both p16<sup>INK4a</sup> and p19<sup>ARF</sup>, p21<sup>Cip1</sup>, E2F-1, and E2F-2. Remarkably, the degree of apoptosis induced by 5-aza-dC in cells lacking the above-mentioned cell-cycle regulators was very modest compared to the apoptotic response induced in p53-null cells (Figure 3a). These data suggest that p53 plays a critical role in providing protection against 5-aza-dC. To further substantiate this, we examined p53 in wild-type MEFs after exposure to 5-aza-dC. We observed increased protein levels of p53 after treatment with 5-aza-dC (Figures 3b, c). Moreover, we also obtained evidence for the functional activation of p53 in response to 5-aza-dC as inferred from the upregulation of the well-established p53-transcriptional targets p21<sup>Cip1</sup> and Bax (Figure 3d).

The above results prompted us to analyse the effects of 5-aza-dC on the cell cycle of p53-proficient cells. 5-Aza-dC resulted in a significant and specific accumulation of cells in the G2/M compartment of the cell cycle (see example in Figure 4a). Quantification of the relative change induced by 5-aza-dC in the percentage of G2/M cells unequivocally showed that the G2/M arrest is specifically dependent on p53, but not on other cell-cycle regulators examined, namely, p19<sup>ARF</sup>, p16<sup>INK4a</sup>, p21<sup>Cip1</sup>, E2F-1, and E2F-2 (Figure 4b). In the case of p53-null MEFs, cells were unable to accumulate in G2/M in response to 5-aza-dC and, in fact, G2/M cells disappeared consistent with the occurrence of apoptotic death, as shown above. All together, these data compellingly indicate that, in p53-proficient cells, 5-aza-dC activates p53 enforcing a p53-dependent G2/M cell-cycle arrest.



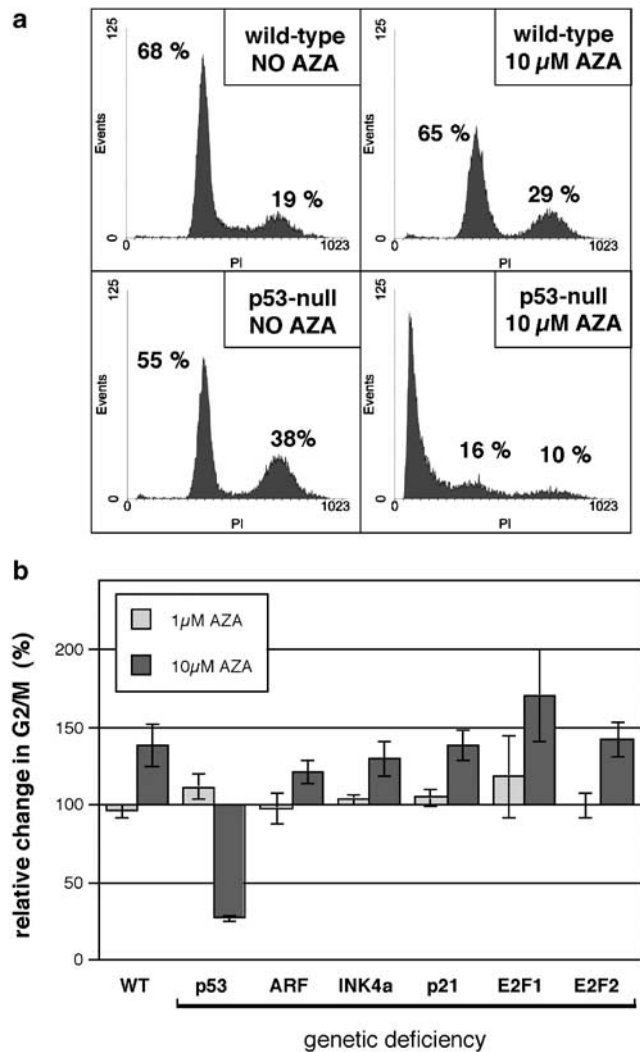
**Figure 2** Relative effect of demethylating drugs on apoptosis and total 5-methyl-cytosine (5mC) content. (a) Cell death was measured as the percentage of cells with sub-G1 DNA content, 6 days after exposure of cells to the indicated treatments. Treatments were as follows: 1  $\mu$ M or 10  $\mu$ M 5-aza-dC (AZA); 100  $\mu$ M zebularine (ZEB); 1 mM *S*-adenosyl-homocysteine (ADO); and 1 mM procainamide (PRO). Each value corresponds to the average and s.d. of three parallel assays, each one with cell cultures from independent embryos. (b) Total genomic content of 5mC after treatment with demethylating drugs. 5mC content was measured in relation to total cytosine content by high-performance capillary electrophoresis in primary MEFs treated for 3 days with the indicated drugs (see legend to part (a) of this figure). The values correspond to triplicate measurements of one culture of MEFs for each genotype



**Figure 3** Specific role of p53 in response to 5-aza-dC. (a) Primary MEFs of the indicated genotype were treated with 5-aza-dC, for 6 days. Some genotypes are abbreviated as follows: wild type (WT), deficiency in p19<sup>ARF</sup> (ARF), deficiency in both p19<sup>ARF</sup> and p16<sup>INK4a</sup> (INK4a), deficiency in p21<sup>Cip1/Waf1</sup> (p21). Cell death was measured as the percentage of cells with sub-G1 DNA content. The values correspond to the average and s.d. of three parallel assays, each one with independently derived cultures of MEFs. (b) Protein levels of p53 in primary wild-type MEFs treated with 5-aza-dC under the indicated conditions. (c) Protein levels of p53 in cells of the indicated genotype, treated with 10  $\mu$ M 5-aza-dC during the indicated times. p53 was determined by immunoblot. The same membranes were subsequently probed with anti- $\beta$ -actin, as a loading control. (d) Functional activation of p53. The protein levels of p53 are shown together with those of the p53-transcriptional targets p21<sup>Cip1</sup> and Bax. Note the presence of nonspecific bands crossreacting with the anti-p53 antibody

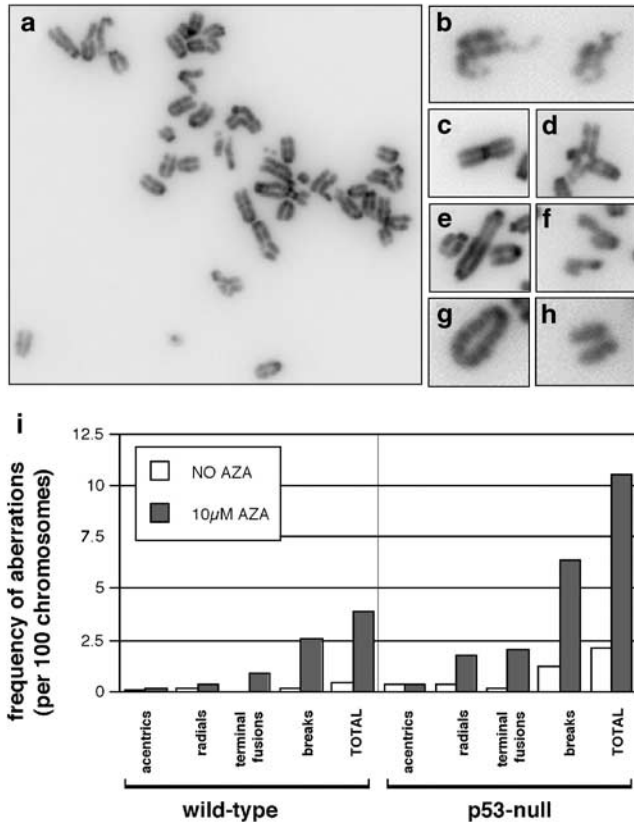
#### 5-Aza-dC induces severe chromosomal damage in p53-deficient MEFs

From the data presented above, we hypothesized that the absence of p53, by failing to enforce a G2/M cell-cycle arrest, could be permissive with regard to the accumulation of genomic instability triggered by 5-aza-



**Figure 4** 5-Aza-dC triggers a p53-dependent cell-cycle arrest at G2/M. (a) Cell-cycle profile of wild-type and p53-deficient MEFs after treatment with 5-aza-dC. Cultures were treated with 10  $\mu$ M 5-aza-dC for 6 days and were subsequently examined by flow cytometry. The numbers indicate the percentage of cells at the G0/G1 and G2/M compartments. (b) Relative change in the percentage of cells at G2/M upon treatment with 5-aza-dC of primary MEFs of different genotypes. Cultures were treated for 6 days with the indicated concentrations of 5-aza-dC and then examined by flow cytometry. The values correspond to the average and s.d. of three parallel assays, each one with independently derived cultures of MEFs

dC. To test this possibility, we analysed metaphasic spreads 3 days after initiating the treatment with 5-aza-dC, that is, before cell death was apparent (Figure 1). Inspection of metaphases clearly showed that 5-aza-dC induced a variety of chromosomal aberrations (see examples in Figures 5a–h). Quantification of chromosomal aberrations indicated the existence of a basal level of chromosomal instability in p53-deficient MEFs (Figure 5i), as has been previously described (Harvey *et al.*, 1993; Purdie *et al.*, 1994). Interestingly, treatment with 5-aza-dC resulted in a dramatic accumulation of chromosomal damage in p53-null MEFs, with more



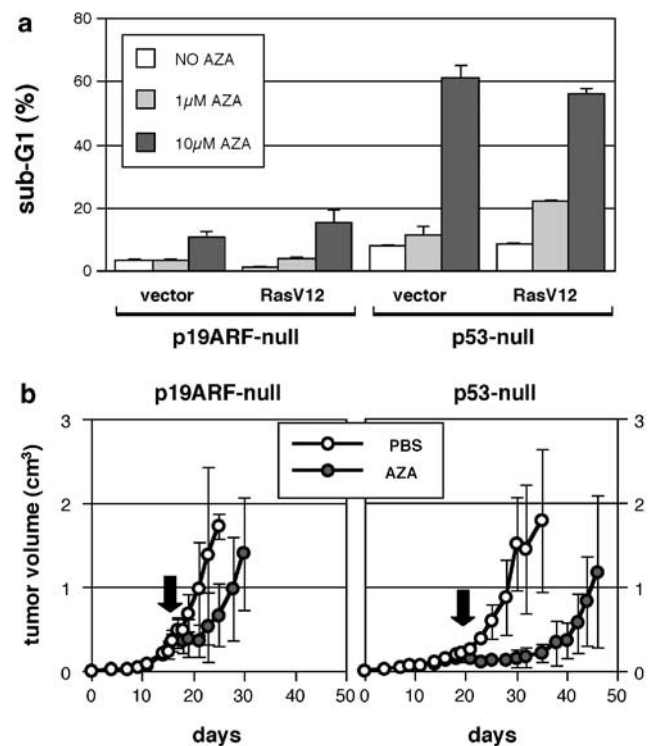
**Figure 5** Chromosomal aberrations triggered by 5-aza-dC in p53-deficient cells. (a) Example of a metaphase spread from p53-deficient MEFs treated with 5-aza-dC. Cells were treated with 10 μM 5-aza-dC for 2 days, and then were allowed to recover for 1 day before addition of colcemid (see Materials and methods). Numerous aberrations can be observed, such as chromatid breaks, chromatid fusions, and complex chromosome fusions. Detailed examples of frequent aberrations are shown in the following figures: (b) chromosomes partially decondensed; (c) chromosome fusion through the p-arms (Robertsonian type); (d) chromatid association (quadriradial); (e) chromosome fusion through the q-arms (dicentric); (f) chromatid breaks; (g) Intrachromosomal fusion of chromatids (ring chromosome); (h) chromosome without centromere (acentric); and (i) quantification of the most common types of aberrations observed in metaphases from wild-type and p53-deficient MEFs, treated or not with 5-aza-dC, as indicated. Terminal fusions include dicentrics, Robertsonian-like fusions, rings, and other complex fusions. Breaks include both chromosome and chromatid breaks. A total of 50 metaphases were scored for each condition. The numbers indicate the frequency per 100 chromosomes

than 10% of the chromosomes showing cytogenetic evidence of damage (Figure 5i). These results suggest that the apoptotic response observed in p53-deficient cells treated with 5-aza-dC is due to the accumulation of genetic aberrations, followed by a failure to arrest the cell cycle, which finally results in mitotic catastrophe.

*Neoplastic transformation does not abolish the hypersensitivity of p53-deficient cells to 5-aza-dC*

We have evaluated the potential relevance of our results in the context of neoplastically transformed cells and,

also, in the context of experimental tumors. MEFs deficient in the ARF-p53 pathway are known to be fully permissive to the oncogenic potential of constitutively activated Ras (Serrano *et al.*, 1997; Palmero *et al.*, 1998). Accordingly, ARF- and p53-deficient primary MEFs were retrovirally transduced with H-RasV12 to generate homogeneous populations of neoplastic cells that contained or not p53, respectively. Remarkably, neoplastic p53<sup>-/-</sup>;RasV12 MEFs retained a similar degree of sensitivity to 5-aza-dC as p53<sup>-/-</sup> MEFs transduced with an empty vector (Figure 6a). In contrast, neoplastic ARF<sup>-/-</sup>;RasV12 MEFs showed a residual degree of apoptosis similar to that of vector-transduced ARF<sup>-/-</sup> MEFs (Figure 6a). Thus, despite the profound deregulation exerted by RasV12, cells were still hypersensitive to 5-aza-dC in a manner that was strictly dependent on the absence of p53.



**Figure 6** Neoplastic cells and tumors lacking p53 are hypersensitive to 5-aza-dC. (a) Primary MEFs of the indicated genotype were retrovirally transduced with an empty vector or with a retrovirus expressing the oncogenic allele H-RasV12. Cell death was measured as the percentage of cells with sub-G1 DNA content, 6 days after exposure of cells to the indicated concentrations of 5-aza-dC. The values correspond to the average and s.d. of three parallel assays, each one with independently derived cultures of MEFs. (b) Therapeutic responsiveness of genetically defined tumors growing in immunocompromised mice. MEFs p53<sup>-/-</sup> or ARF<sup>-/-</sup> were neoplastically transformed by retroviral transduction of H-RasV12 (see part (a) of this figure). These cells were injected subcutaneously into nude mice, and the growth of tumors was scored every 2–3 days. Eight mice were injected in both flanks with one culture of MEFs of each genotype. When the volume of the tumors reached approximately 250 mm<sup>3</sup> (day 15 for ARF<sup>-/-</sup> and day 18 for p53<sup>-/-</sup>; marked with an arrow in the figures), half of the mice were injected intraperitoneally with 15 mg/kg of 5-aza-dC (see Materials and methods for further details) and the other half with PBS, as indicated

To further extend these observations, we attempted to recapitulate the hypersensitivity of p53-null cells in the context of experimental tumors. The above-described RasV12-transformed cells, lacking either ARF or p53, were injected subcutaneously into immunodeficient mice. The injected cells were allowed to graft and form a sizeable tumor (with an estimated volume of approximately 250 mm<sup>3</sup>), which was reached by both types of cells in about 15 days (Figure 6b). At this time, mice received a single treatment of 5-aza-dC intraperitoneally. This treatment was chosen in accordance to a previous study that determined the highest dose of 5-aza-dC that was not toxic in mice, and that was subsequently shown to induce DNA demethylation (Plumb *et al.*, 2000). Treatment with 5-aza-dC was able to completely arrest the growth of the p53-deficient tumors for approximately 25 days. In contrast to this, 5-aza-dC only delayed the growth of the ARF-deficient tumors for about 5 days, probably due to the 20% residual apoptosis observed *in vitro* (Figure 6a). We conclude that the absence of p53 significantly increases the sensitivity to 5-aza-dC even in the context of an animal tumor and at drug doses that do not manifest toxicity.

## Discussion

In this work, we have examined the impact of p53 on the cellular response to 5-aza-dC. p53 has been classically described as a mediator of the cytotoxicity of many genotoxic drugs by promoting either cell-cycle arrest or apoptosis (Kuerbitz *et al.*, 1992; Lee and Bernstein, 1993; Lowe *et al.*, 1993). However, and in opposite direction, there is another factor that is becoming increasingly recognized. In particular, cells deficient in p53, by failing to arrest the cell cycle in response to genotoxic agents, may continue proliferation and enter into a mitotic catastrophe (Hendry and West, 1997; Ianzini and Mackey, 1998; Roninson *et al.*, 2001; Gudkov and Komarova, 2003). The concept of sensitization to apoptosis by the absence of p53 is illustrated by a number of examples, such as in response to cisplatin (Hawkins *et al.*, 1996; McKay *et al.*, 2001), taxol (Hawkins *et al.*, 1996), UV (Lackinger and Kaina, 2000; Smith *et al.*, 2000; McKay *et al.*, 2001), or  $\gamma$ -radiation (Lips and Kaina, 2001). Here, we have extended this concept to the demethylating drug 5-aza-dC, which shows an exceptionally high selectivity for p53-deficient cells. When compared to a number of other chemotherapeutic and demethylating drugs, 5-aza-dC showed the highest selectivity ratio for triggering cell death in p53-deficient cells relative to wild-type cells. Moreover, this is a property that seems exclusive of p53-deficient cells, not being present in cells deficient in other cell-cycle regulators. Finally, the selectivity for p53-deficient cells is retained even in the context of cells neoplastically transformed with oncogenic Ras. Oncogenic transformation by Ras is known to result in a profound deregulation of cellular homeostasis that

affects essentially all aspects of cellular biology (Zuber *et al.*, 2000). Having this in mind, it is highly suggestive the fact that the functional status of p53 determines the sensitivity to 5-aza-dC in cellular contexts that are dramatically different.

In agreement with the concept that p53-deficiency results in catastrophic proliferation in the face of rampant genomic damage, we have observed extremely high levels of chromosomal damage in p53-null cells treated with 5-aza-dC. In contrast, p53-proficient cells react to the presence of 5-aza-dC by activating p53 and enforcing a p53-dependent G2/M arrest. Previous investigators have also reported that 5-aza-dC results in p53 activation in colon cancer cell lines in association with proliferative arrest (Karpf *et al.*, 2001). In this latter case, however, the presence of p53 correlated with a lower cellular viability (Karpf *et al.*, 2001) and this is in contrast to our data where the presence of p53 correlated with apoptotic death. This discrepancy is most likely due to differences between MEFs and the colon cancer cell lines used in Karpf *et al.* (2001). In any case, both sets of evidence make clear that further studies are needed in therapeutically relevant systems, such as myeloid leukemia, to ascertain the potential relevance of p53 in the response to 5-aza-dC.

Regarding the mechanisms underlying the chromosomal damage produced by 5-aza-dC, we have observed a significant global hypomethylation of the genome. It has been reported that hypomethylation due to genetic manipulation of DNMT1 results in genetic instability (Chen *et al.*, 1998; Eden *et al.*, 2003; Gaudet *et al.*, 2003) and p53-dependent apoptosis (Jackson-Grusby *et al.*, 2001; Stancheva *et al.*, 2001). However, demethylation by itself cannot simply explain our results. First, p53-deficient cells are demethylated to the same extent as p53-proficient cells. Previously, it has been shown that 5-aza-dC has a similar demethylating activity on a variety of cancer cell lines (Paz *et al.*, 2003). Here, we have extended this observation by demonstrating, in genetically controlled cells, that the status of p53 does not influence the demethylating activity of 5-aza-dC. Second, low concentrations of 5-aza-dC (1  $\mu$ M) are very effective in inducing demethylation, yet do not result in significant cell death of p53-deficient cells. For these reasons, hypomethylation cannot explain directly the observed apoptotic effect. Also, it is known that 5-aza-dC exerts an important part of its cytotoxic effects through the formation of protein-DNA adducts with the DNMTs (Juttermann *et al.*, 1994). As a result, cells treated with 5-aza-dC are subject to a severe DNA damage, which in p53-proficient cells triggers a p53-dependent G2/M checkpoint preventing further proliferation of the damaged cells. In the case of p53-deficient cells, two factors concur to aggravate the consequences of the DNA damage induced by 5-aza-dC. On one hand, p53-deficient cells are known to be ineffective in various aspects of DNA repair (Bernstein *et al.*, 2002; Lin *et al.*, 2003); on the other hand, p53-deficient cells fail to execute the G2/M checkpoint. All things considered, the most plausible mechanism to explain the apoptotic

response triggered by 5-aza-dC in p53-deficient cells is that cells are subject to multiple levels of genomic damage and, by failing to arrest their proliferation, enter into a mitotic catastrophe.

The greatest therapeutical success of 5-aza-dC has been observed in the treatment of acute myeloid leukemia, advanced myelodysplastic syndromes, and chronic myeloid leukemias in blastic phase (Santini *et al.*, 2001), and moderate responses have been observed in a variety of solid tumors (Santini *et al.*, 2001). It is interesting to note that loss of p53 is highly prevalent in advanced myelodysplastic syndromes (Mori *et al.*, 1995; Tang *et al.*, 1998), and in the blastic phase of chronic myeloid leukemia (Lanza and Bi, 1995; Stuppia *et al.*, 1997). However, specific trials to evaluate the efficacy of 5-aza-dC on p53-proficient and p53-deficient tumors are still lacking. As a first approximation towards this end, we have examined the response of genetically controlled tumors to 5-aza-dC. We have observed that treatment with a nontoxic dose of 5-aza-dC had a measurable therapeutic response against p53-deficient tumors, but not p53-proficient tumors (which in our case were ARF-deficient). While we have not measured directly the concentration of 5-aza-dC achieved in the plasma of the mice used in these experiments, it is worth to mention that the concentration used in our cell culture assays (10  $\mu$ M 5-aza-dC) can be conceivably achieved with the current doses of 5-aza-dC used in clinical trials. In particular, these trials have typically employed doses in the range of 100–1000 mg/m<sup>2</sup> of body surface area (Santini *et al.*, 2001), and it has been demonstrated that at the lowest dose (100 mg/m<sup>2</sup>), the peak concentration of 5-aza-dC in plasma is of 2  $\mu$ M (van Groeningen *et al.*, 1986). Finally, there is evidence of synergism between 5-aza-dC and other chemotherapeutic treatments (Plumb *et al.*, 2000; Santini *et al.*, 2001). With all the due concerns about extrapolating our data to a clinical context, we would like to raise the hypothesis that the absence of functional p53 may contribute to the clinical efficacy of 5-aza-dC.

In summary, our results support a model by which 5-aza-dC exerts its antitumoral activity mainly through the induction of severe chromosomal damage. In the case of p53-proficient cells, treatment with 5-aza-dC results in G2/M arrest. In contrast, in p53-deficient cells, the above-mentioned G2/M arrest cannot be implemented and cells enter into catastrophic mitosis followed by cell death. These observations set the stage to evaluate, in clinically relevant settings, the impact that the status of p53 might have on the efficacy of 5-aza-dC.

## Materials and methods

### Cell culture and drug treatment

Primary mouse fibroblasts (MEFs) were derived from E13.5 embryos obtained from the following colonies of mice: *wild type*, *p53*<sup>-/-</sup> (Jacks *et al.*, 1994), *p21*<sup>Cip1/Waf1</sup><sup>-/-</sup> (Brugarolas *et al.*, 1995), *ARF*<sup>-/-</sup> (Kamijo *et al.*, 1997), *INK4a/ARF*<sup>-/-</sup> (Serrano *et al.*, 1996), *E2F-1*<sup>-/-</sup> (Field *et al.*, 1996), and *E2F-2*<sup>-/-</sup> (Murga *et al.*, 2001). Mice were maintained at the Spanish National

Center of Biotechnology, Madrid, in a mixed genetic background C57Bl/6;129/Sv for *ARF*<sup>-/-</sup>, *E2F-1*<sup>-/-</sup>, and *E2F-2*<sup>-/-</sup>, or in a pure C57Bl/6 for wild type, *p21*<sup>-/-</sup>, *p53*<sup>-/-</sup>, and *INK4a/ARF*<sup>-/-</sup>. MEFs were prepared as described (Palmero and Serrano, 2001) and maintained in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% FBS (Sigma). 5-Aza-dC (Sigma) was solubilized in complete culture media and stored at -20°C up to 2 months. Cells were plated at a density of 8 × 10<sup>5</sup> per 10 cm diameter plate (or equivalent density when using a different plate size) and treated as indicated for each experiment (see legends to figures).

### Western blot

Whole-cell extracts were prepared by lysis of cells in a buffer containing 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% NP-40, 1 mM DTT, 1 mM PMSF, 10 mM NaF, and 4  $\mu$ g/ $\mu$ l of protease inhibitors cocktail (aprotinin, leupeptin, and pepstatin). Samples containing equal amounts of protein were separated on 12% SDS polyacrylamide gels and blotted onto nitrocellulose membranes. Membranes were blocked in phosphate-buffered saline (PBS) containing 0.2% Tween-20 and 5% powdered milk before the addition of antibodies. The primary antibodies used were: anti-p53 (Ab-1, Oncogene Research Products, 1:200), anti-p21 (C19, Santa Cruz, 1:500), anti-Bax (P-19, Santa Cruz, 1:200), and anti- $\beta$ -actin (AC-15, Sigma, 1:10000). After incubation with horseradish peroxidase-coupled secondary antibodies (anti-rabbit, Amersham, 1:1000 or anti-mouse, Dako, 1:1000, as it corresponds), signals were detected by chemiluminescence using ECL (Amersham).

### Detection of apoptosis

Apoptosis was measured using a variety of assays, which include the quantification of cells with sub-G1 DNA content, annexin-V reactivity, and caspase-3-like activity. In all cases, unattached and attached cells were harvested for the analysis. For sub-G1 DNA content, cells were collected by trypsinization, washed with PBS and stained with propidium iodide using the DNA-Prep reagent (Coulter Corporation). Cells with sub-G1 DNA content were quantified by flow cytometry using an Epics XL cytometer (Coulter Corporation). For annexin-V reactivity, cells were harvested by trypsinization, washed with PBS and incubated in annexin-V binding buffer (10 mM HEPES pH 7.4, 140 mM NaCl, 2.5 mM CaCl<sub>2</sub>, 1% BSA). Cells (1 × 10<sup>6</sup>) suspended in binding buffer were stained with fluorescently labelled annexin-V and propidium iodide (Annexin V-FITC Kit, Immunotech). Annexin-V-positive cells were quantified by flow cytometry in an Epics XL cytometer (Coulter Corporation). For caspase-3-like activity, cells were synchronized in G0 by serum deprivation (0.1% FBS) for 3 days. After this time, cells were stimulated with complete medium (10% FBS) in the presence of 10  $\mu$ M 5-aza-dC for the indicated times. Cells were harvested with EDTA, and the caspase-3 activity was determined as described (Ruiz-Vela *et al.*, 2002). Briefly, cytosolic extracts were diluted in caspase buffer (25 mM HEPES pH 7.5, 0.1% CHAPS, 10% sucrose, 10 mM DTT, and 0.1 mg/ml ovalbumin) and incubated with 10  $\mu$ M of the fluorescent substrate Ac-DEVD-AMC (acetyl-asp-glu-val-asp-7-amino-4-methylcoumarin). Cleaved substrate was determined by C18 reverse-phase HPLC using fluorescence detection.

### Determination of the 5mC content

5mC content was determined by high-performance capillary electrophoresis, as previously described (Fraga *et al.*, 2002).

Briefly, genomic DNA samples were boiled, treated with nuclease P1 (Sigma), for 16 h, at 37°C, and with alkaline phosphatase (Sigma) for an additional 2 h, at 37°C. After hydrolysis, the total content of cytosine and 5mC was measured by capillary electrophoresis using a P/ACE MDQ system (Beckman-Coulter). The relative content of 5mC was expressed as the percentage with respect to the total cytosine content (methylated and nonmethylated).

#### Chromosomal aberrations

Cells were treated with 10  $\mu$ M 5-aza-dC for 48 h and then were allowed to recover for 24 h. Colcemid (Karyomax, Gibco) was then added to a final concentration of 0.1  $\mu$ g/ml and incubated for 4 h. Cells were harvested by trypsinization, treated with hypotonic buffer (0.03 M sodium citrate), for 5 min, and fixed with methanol/acetic acid (3:1). Slides were prepared by standard cytogenetic techniques and chromosomes were stained with DAPI. About 50 metaphases were scored for each experimental condition.

#### Retroviral transduction

Retroviral transduction was done as described (Abad *et al.*, 2002). In brief,  $6 \times 10^6$  293T cells were cotransfected by calcium phosphate precipitation with 15  $\mu$ g of the helper vector pCL-Eco (that expresses the viral proteins gag-pol and the ecotropic envelope) and the retroviral vector pLPC (encoding puromycin resistance) either empty or carrying H-RasV12. Retroviral supernatants were collected and added to the recipient MEF cultures following standard procedures (Palmero and Serrano, 2001), and 48 h after, infected cells were

selected with 2  $\mu$ g/ml puromycin for 3 days. Cells were then pooled and analysed.

#### Tumorigenesis assay

Neoplastically transformed MEFs (*p53*<sup>-/-</sup>/H-RasV12 or *ARF*<sup>-/-</sup>/H-RasV12) were harvested with trypsin/EDTA and resuspended in PBS. Cells ( $5 \times 10^5$ ) were injected subcutaneously into both flanks of female nude mice (8 weeks old). Tumor volumes were estimated from caliper measurements of tumor length (*L*) and width (*W*) according to the following formula:  $(L \times W)^2/2$ . When tumors reached a volume of about 250 mm<sup>3</sup>, mice were treated with 5-aza-dC according to a previously published protocol (Plumb *et al.*, 2000). Specifically, the treatment consisted in three intraperitoneal injections, at intervals of 3 h, with a dose of 5 mg/kg per injection (total 15 mg/kg).

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